

## THE IMMUNITY INDEX METHOD OF TESTING ANTIGENIC VALUES,

WITH ILLUSTRATIONS OF ITS USE IN SHOWING THE EFFECTS OF BROTH, FORM-ALDEHYDE, PHENOL, SODIUM RICINOLEATE, ALUM, TURPENTINE, TOLUOL, ACID PRECIPITATION AND HEAT ON DIPHTHERIA TOXOID; THE INTERFERENCE OF DYE BLOCKADE WITH ANTIGENIC RESPONSE AND OF ONE RESPONSE WITH ANOTHER; THE DISSOCIATION OF TOXIN-ANTITOXIN MIXTURES ON DILUTION.

A. T. GLENNY and HILDA WADDINGTON.

*Wellcome Physiological Research Laboratories, Beckenham, Kent.*

THE immunity index method of testing the antigenic values of diphtheria prophylactic was established by Glenny Allen and Hopkins (1923). This method without any modification is used in these laboratories as the routine method of testing the antigenic efficiency of most prophylactic material. Recently there has been introduced the rapid index method for determining the value of those preparations able to induce immunity in a shorter time than can be estimated by the routine method.

In the *routine method* guinea-pigs are Schick tested at weekly intervals commencing three weeks after the injection of the antigen under test. If the animals give a negative reaction to the first Schick injection the immunity index is recorded as 1; if positive to the first and negative to the second the index is 2 etc. In the *rapid method* the interval between injection of the antigen and the first Schick test is cut down to 10 days and the Schick injections are repeated every two days instead of weekly. The index is given as the number of days taken by the animal to become immune and not the number of Schick injections as in the routine method.

The immunity index essentially measures potential immunity rather than the actual amount of antitoxin produced. A single injection of a Schick dose of toxin must be regarded as a relatively efficient secondary stimulus but of almost negligible value as a primary stimulus. The secondary stimulus effect of the injection of Schick toxin, demonstrated by Glenny and Allen (1922), increases the rate at which the test guinea-pigs become immune so that an animal that is Schick negative at the second test would not necessarily have reached that level of immunity if the first Schick test has been omitted. In this way the immunity index method magnifies the antigenic response and far smaller amounts of antibody can be detected than by any method of measurement of tolerance of toxin or antitoxin production following

a single injection. The secondary stimulus effect of Schick toxin has been confirmed in human immunisation by O'Brien (1926) Kellogg and Stevens (1927) Opitz (1927) and Harries (1927).

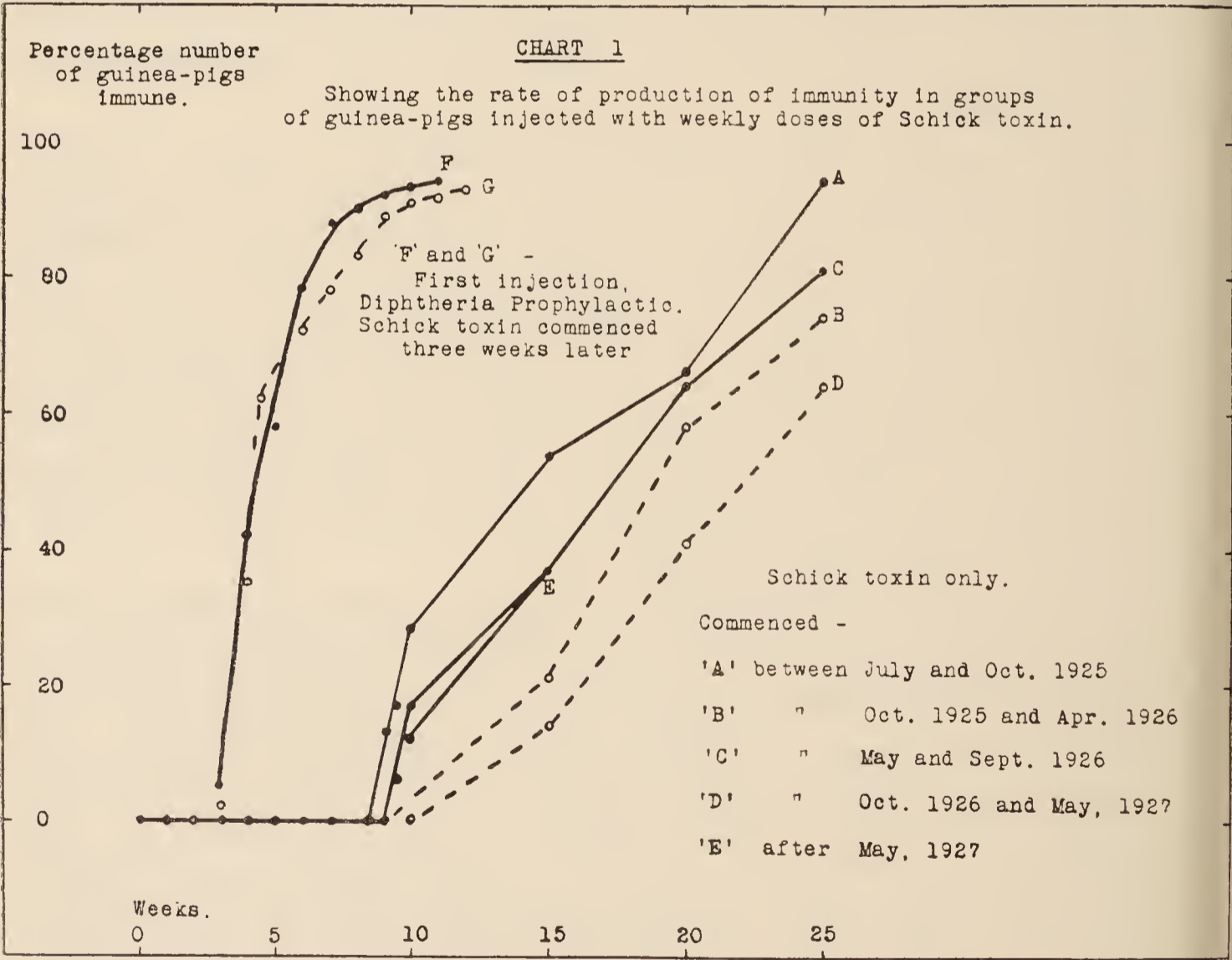
Controls.

The immunity index method is controlled by Schick testing at weekly intervals guinea-pigs that have not received a primary stimulus. Considerable variation occurs in the time taken by such control guinea-

TABLE I.

*Showing the seasonal variation found in the time taken by normal guinea-pigs to be immunised by weekly injections of a Schick dose of toxin.*

Date of commencement.	Number of guinea-pigs.	Percentage number of guinea-pigs immunised in—			
		10 weeks.	15 weeks.	20 weeks.	25 weeks.
14th July to 6th October 1925 . . . . .	60	28	54	66	94
13th October to 20th April 1926 . . . . .	63	0	21	58	74
11th May to 14th September 1926 . . . . .	35	17	37	65	81
5th October to 3rd May 1927 . . . . .	49	0	14	41	64
24th May . . . . .	(24)	16	37	75	83



pigs to be immunised and there appears to be some seasonal variation depending probably upon the general condition of health at different times of the year. Guinea-pigs have been immunised to the Schick

negative level within 8 weeks by weekly injections of Schick toxin, while other guinea-pigs have remained Schick positive after two years of this treatment. The average time is between 15 and 20 weeks. Commencing in July 1925, control tests were made at regular intervals and the results from over 200 guinea-pigs are summarised in table I which, with chart 1, shows that guinea-pigs are more readily immunised by small doses of toxin when immunisation is commenced in the summer or early autumn; at this period of the year fresh green food is more plentiful and this may afford a possible explanation of seasonal variation.

In a similar way the rapid index method has been controlled by injecting normal guinea-pigs three times a week with Schick toxin. Over 20 such injections are needed on the average to immunise guinea-pigs by Schick toxin alone. The most rapid immunisation was that of one guinea-pig that became Schick negative after 13 injections. For this reason no rapid immunity index experiments are carried beyond 10 Schick tests, the guinea-pigs being discarded at this stage.

#### *Uniformity of results.*

Individual guinea-pigs vary in their power of response to an antigen and a number of animals must be used for each test; repeat tests made upon groups of 4 to 6 guinea-pigs on each occasion give fairly consistent results. Table II shows the results of 8 consecutive tests upon the same toxin-antitoxin mixture repeated every 3 weeks. On each occasion the majority of guinea-pigs gave an index of 2 or 2 to 3.

TABLE II.

*Showing the immunity index of groups of 4 to 6 guinea-pigs each injected with 1.0 c.c. of the same toxin-antitoxin mixture.*

Date of test	Immunity indices.					
13th July . . .	2	2	3	3	4	5
3rd August . . .	2	2	3	3	4	4
24th August . . .	2	2	2	3	4	4
14th September . . .	2	2	2	2	5	over 10
5th October . . .	1	2	2	2	5	...
26th October . . .	2	2	2	4	5	over 10
16th November . . .	2	2	2	5	...	...
7th December . . .	2	2	2	5	...	...

A close analysis of similar results extending over two years has shown no evidence of seasonal variation. It is suggested that any possible seasonal variation in antitoxic response to such stimuli that induce potential immunity in about three weeks affects the degree of active response rather than the latent period elapsing before potential immunity is acquired.

It is usually found that animals in poor condition are less readily immunised and, although it is not always so, as a general rule the

guinea-pigs in any group showing the poorest index are those that increase least in weight during the period of observation.

A total of 227 guinea-pigs of various weights varying from 210 to 870 grms. and obtained from different sources of supply were injected with 5 c.c. of the same batch of a certain toxoid on different occasions spreading over 8 months and the guinea-pigs were Schick tested three times a week commencing on the 10th day after injection. An analysis of results is given in table III showing the percentage number of guinea-pigs immunised by the 16th, 19th and 21st days dividing the animals according to sex, commencing weight and general condition as indicated by the loss or gain in weight during the first 10 days after injection. There is no evidence to show that there is any difference in response between bucks and does but for a given dose of antigen the immunity response was quicker with smaller than with larger guinea-pigs. The effect of condition is quite definite. Guinea-pigs losing in weight showed only 57 per cent. negative by the 21st day while those that gained steadily in weight gave 66 per cent. negative by the 16th day.

TABLE III.

*Showing the percentage number of guinea-pigs with a rapid index of 16, 19 and 21 days divided according to sex, commencing weight and general condition.*

Index in days.	Bucks.	Does.	Commencing weight in grms.			Change in weight in 10 days.		
			300.	300-500.	500.	Loss.	Moderate gain.	Large gain.
16	47	41	52	36	38	28	44	66
19	63	79	82	65	62	38	46	94
21	77	85	89	78	71	57	88	94

#### *Interpretation of results.*

In order to interpret any immunity index figures it was found necessary to work out the influence of varying the time between injection of the antigen and commencement of Schick testing. The figures obtained made it possible to differentiate between the time when the animals under test were potentially immune, that is able to produce a secondary stimulus response to a subsequent injection, and actively immune, that is Schick negative.

A number of guinea-pigs were injected with four different doses of diphtheria toxoid and were then Schick tested at weekly intervals, some of each group commencing one week and some two three and four weeks etc. after the initial injection. Table IV records the results obtained.

TABLE IV.

Showing the first negative Schick test after the injection into guinea-pigs of different doses of toxoid followed by weekly Schick tests commencing at different times after the initial injection.

Number of Lf doses of toxoid injected.	Interval between primary stimulus and Schick test in weeks.					
	1.	2.	3.	4.	6.	13.
0·5	4, 5, 6, 6	3, 4, 5 >10	3, 4, 7	2, 2, 7 >10	2, 5, 7, 9	1, 1, 2 >7
2	2, 3, 3, 3	2, 2, 2, 2	2, 2, 2, 4	1, 1, 2, 4	1, 1, 1	1, 1, 1
16	2, 2, 2	1, 2, 2, 2	1, 1, 1, 1	1, 1, 1	1, 1, 1, 1	1, 1
50	2, 2, 2	1, 1, 1, 1	1, 1, 1, 1	1, 1, 1, 1	1, 1, 1, 1	1, 1

The following conclusions can be drawn from these results :

	Lf doses.	Number of weeks to become potentially immune.	Number of weeks to become actively immune, Schick negative.	
	0·5	4	6 to 13 or more	
	2	2	4 to 6	
	16	1	3	
	50	0	2	

A similar experiment was made to determine the influence of the time of starting Schick testing upon the rapid immunity index. The same batch of toxoid was used as in the previous experiment. The results are given in table V.

TABLE V.

Showing the number of days taken for guinea-pigs to reach the Schick negative level after the injection of different doses of toxoid followed by Schick toxin given every two days commencing at different times after the initial injection.

Number of Lf doses of toxoid injected.	Interval between primary stimulus and first Schick test in days.									
	6.	8.	10.	12.	14.	16.	19.	24.	28.	42.
2	14	14	14, 16	19, 19	19	19	26	24	28	42
	19	16	19, 19	19, 21	23	19	26	30	28	47
	21	19	19, 19	19, 23	23	21	26	30	30	49
	23	21	21, 30	21, 30	...	21	28	30	37	58
16	14	12	12, 16	14	14	16	19	...	...	...
	16	14	14, 19	14	14	16	19	...	...	...
	16	14	14, 19	14	16	16	over 30	...	...	...
	21	19	21, 21	16	21	26	...	...	...	...

Summarising the results with 16 Lf doses we find that—

No guinea-pig was Schick negative when first tested before the 14th day.

2	out of 4	were	„	„	on	14th	„
3	„	4	„	„	„	16th	„
2	„	3	„	„	„	19th	„

We may assume therefore that this dose of toxoid produces immunity above the Schick level in 14 to 16 days in the majority of guinea-pigs injected. Analysing the results of the animals Schick tested before the 14th day we find that among 20 guinea-pigs injected two were immune by the 12th day, eight by the 14th day and four by the 16th day. As these results are comparable with those obtained when the guinea-pigs were injected first on the 14th and 16th day, we may conclude that the earlier Schick doses were ineffective in accelerating the production of immunity. If we consider that 2 days are insufficient in which to produce an increase in immunity after a secondary stimulus but that in 4 days a sufficient secondary response should occur, then we must conclude that few animals are potentially immune by the 10th day after the injection of 16 Lf doses of the toxoid. It may further be concluded that when tests are made upon a product giving a rapid immunity index of 14 to 16 days the apparent index is not affected by the date at which Schick testing is commenced.

Some indication of the degree of potential immunity at different dates after the primary injection of 2 Lf doses may be obtained by summarising the results given in table V as follows:—

Starting on the 6th day the guinea-pigs were immune in 13 or more days later.								
”	”	8th	”	”	”	10	”	”
”	”	10th	”	”	”	8	”	”
”	”	12th	”	”	”	8	”	”
”	”	14th	”	”	”	6	”	”
”	”	16th or after	”	”	”	3 to 5 more days.		

The rapidity with which guinea-pigs acquired the power to respond (potential immunity) was also shown in another experiment. Guinea-pigs were injected with 0·2 c.c. (2 Lf doses) of a certain toxoid and then with 0·01 c.c. of the same toxoid 7, 10 or 14 etc. days later and were Schick tested daily starting two days after the second injection of toxoid. When the interval between the primary and the secondary stimulus was 17 or 21 days it was found that the first Schick test

TABLE VI.

*Showing the time taken for guinea-pigs to become Schick negative after an injection of 0·01 c.c. toxoid given at different intervals of time after a primary stimulus.*

	Interval between primary and secondary stimulus in days.							
	7.	10.	14.	17.	21.	28.	56.	84.
Interval in days between secondary stimulus and Schick negative response.	7	3	3	(2)	(2)	0	0	0
	7	6	4	(2)	(2)	0	0	0
	10	17	4	4	(2)	2	...	9
	12	...	9	14	3	2	...	9

gave no reaction so that it was impossible to determine whether the guinea-pigs were already immune when they received the second injection of toxoid. Schick testing was therefore commenced on the same day as the second injection of toxoid given 28, 56 or 84 days after the first injection. The results are recorded in table VI.

Accepting a Schick negative reaction in 4 days after the second injection of toxoid as a rapid secondary response indicating potential immunity it will be seen that none of the guinea-pigs was potentially immune when the secondary stimulus was given 7 days after the primary, but the majority of the guinea-pigs were potentially immune when the interval between the two was lengthened to 14 days.

APPLICATION OF METHODS.

1. Qualitative differences in toxoids.

The immunity index methods can be used to demonstrate qualitative differences between toxoids. Table VII shows the immunity index after the injection of 1/4, 1/2, 1 and 2 Lf doses of a number of batches of toxoid of two distinct types.

TABLE VII.

Showing the relation of immunity index to dose injected of two types of toxoid.

Dose injected.	Number of guinea-pigs showing an index of—										Average index.	
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10 or more.	Type A	Type B
¼ Lf Type A .	...	1	4	3	4	...	1	1	1	1	4½	...
„ „ B .	...	...	...	...	...	1	...	2	...	7	...	10
½ Lf Type A .	1	6	5	2	1	1	...	...	...	1	3	...
„ „ B .	...	1	1	1	2	2	...	...	...	3	...	5½
1 Lf Type A .	3	6	5	2	...	...	...	...	...	...	2½	...
„ „ B .	...	3	3	...	1	1	...	...	...	1	...	3
2 Lf Type A .	6	9	...	...	...	...	...	...	...	...	1½	...
„ „ B .	1	3	1	1	2	...	...	...	...	1	...	2

Table VIII gives a similar comparison between the same batches of the two types of toxoid tested by the rapid index method.

In this experiment 10 different batches of toxoid were compared, 6 of type A and 4 of type B. There were slight qualitative differences between individual batches of each type but these differences were smaller than those between any batch of type A and any batch of type B.

It will be seen from these two tables that both the routine index and the rapid index show a consistent difference in the two types of toxoid. Type A was unconcentrated toxoid and type B toxoid

concentrated by the Watson and Wallace (1924) process. It is suggested by these results that nonspecific material harmful to antigenic response is all precipitated by acid; only about 50 per cent. of specific toxoid is precipitated. It follows therefore that the concentration of harmful nonspecific material in comparison with necessary specific antigen is increased by this method of concentration. Moloney and Weld (1925) demonstrated similar qualitative differences between different batches of toxoid. These workers however tested the immunity of the injected guinea-pigs by Schick tests given at irregular intervals and their results are not directly comparable with those given in these tables.

TABLE VIII.

*Showing the relation of rapid immunity index to dose injected of two types of toxoid.*

Dose injected.	Number of guinea-pigs showing an index of:—												Average rapid index in days.	
	10.	12.	14.	16.	19.	21.	23.	26.	28.	30.	Over 30.	Type. A.	Type B.	
1 Lf Type A . .	...	...	2	6	5	4	5	1	2	...	1	16-21	...	
„ „ B . .	...	...	...	...	2	1	2	1	2	1	2	...	26	
2 Lf Type A . .	...	...	5	7	5	4	1	...	...	...	...	16-19	...	
„ „ B . .	...	...	1	1	7	...	1	2	...	...	3	...	19-21	
4 Lf Type A . .	...	2	4	9	7	1	...	...	...	...	...	16	...	
„ „ B . .	...	...	1	5	2	2	2	1	..	...	1	...	19	
8 Lf Type A . .	...	2	11	12	2	5	...	...	...	...	...	14-16	...	
„ „ B . .	...	...	5	4	...	1	...	...	1	2	2	...	16-19	
16 Lf Type A . .	...	9	11	2	5	...	...	...	...	...	...	14	...	
„ „ B . .	...	1	2	3	4	...	...	...	...	...	...	...	16	
32 Lf Type A . .	...	...	...	...	...	...	...	...	...	...	...	...	...	
„ „ B . .	...	...	4	1	1	...	...	...	...	...	...	...	14-16	
64 Lf Type A . .	2	10	4	...	...	...	...	...	...	...	...	12	...	
„ „ B . .	...	1	...	1	1	...	...	...	...	...	...	...	14	

Tables VII and VIII also offer a comparison between the immunity index and the rapid index method.

1 Lf dose of type B gives an average immunity index of	{ 3 and a rapid index of 26 days.
1 „ „ A „ „	{ 2½ and a rapid index of 19 to 21 days.
2 „ „ B „ „	{ 2 and a rapid index of 19 to 21 days.
2 „ „ A „ „	{ 1½ and a rapid index of 16 to 19 days.

Further immunity index experiments have shown qualitative differences among batches of toxoid depending upon the method by

which the broth was made for their preparation. Table IX shows that the injection of 2 Lf doses of toxoid type C is more effective than that of the same dose of type D and type D is better than type E.

TABLE IX.

*Showing the relative antigenic values of 2 Lf doses of batches of toxoid prepared from three types of broth.*

	Number of guinea-pigs showing a rapid index of—		Percentage number under 19 days.
	Under 19 days.	19 days or over.	
Type C .	6	4	60
„ D .	21	41	34
„ E .	1	17	6

A further experiment to confirm these differences was made by diluting 1.0 c.c. of another toxoid in 5.0 c.c. of the three types of toxoid after autoclaving. Table X shows that the inhibitory substances present in certain types of toxoid are not destroyed by autoclaving.

TABLE X.

*Showing the effect of addition of 4.0 c.c. autoclaved toxoid of different types upon the antigenic value of 1.0 c.c. of another toxoid.*

Type of autoclaved toxoid added.	Number of guinea-pigs showing an immunity index of—			Percentage number.	
	2.	3.	Over 3.	2.	3 or under.
Type C .	14	1	3	77	83
„ D .	2	6	3	18	73
„ E .	5	1	5	45	55

The three types of toxoid were prepared, C from bullocks' heart peptone broth, D from tryptic digest of horse muscle, E from tryptic digest of bullocks' heart. In this experiment there appeared to be no correlation between speed of flocculation and antigenic efficiency.

Another experiment recorded in table XI shows the influence of excess of formaldehyde upon the antigenic value of toxoid. There is a correlation between amount of formalin, rate of flocculation and antigenic value; nevertheless modifications of toxoid with excess formaldehyde have high antigenic efficiency even though they fail to flocculate with three different antitoxins A, B and C; one modification with 0.8 per cent. formaldehyde prevents flocculation when added in equal parts to a fresh toxin.

TABLE XI.

*Comparing the antigenic efficiency and speed of flocculation of toxoid prepared from the same original toxin with the addition of varying amounts of formaldehyde.*

Amount of formaldehyde.	Lf values.	Rapid index in days. 1·0 c.c.	Speed of flocculation in minutes—direct titration against 3 sera.			Speed of flocculation in minutes by blend with the original toxin before titration against 3 sera.		
			A.	B.	C.	A.	B.	C.
0·2 per cent.	15	10, 12, 12	40	90	100	25	30	35
0·4 „	15	12, 16	No flocculation in 7 days.			65	120	60
0·6 „	15	14, 14, 16				80	300	240
0·8 „	11	14, 16, 23	„	„	„	360	No flocculation.	

The inhibitory effect of phenol has also been demonstrated by means of the immunity index. The results are shown in table XII.

TABLE XII.

*Comparing the antigenic value of toxoid with and without phenol.*

Dose injected.	Number of guinea-pigs showing an immunity index of—				Percentage number.	
	1.	2.	3.	Over 3.	1.	2 or under.
0·5 Lf no phenol . . .	2	6	1	0	...	89
0·5 Lf with phenol . . .	...	2	1	3	...	33
2·0 Lf no phenol . . .	9	3	1	...	69	...
2·0 Lf with phenol . . .	1	4	1	...	17	...

	Number of guinea-pigs showing rapid index of—					Percentage number.		
	12.	14.	16.	19.	Over 19 days.	12.	14 or better.	16 or better.
2·0 Lf no phenol . . .	...	1	6	3	4	...	7	71
2·0 Lf with phenol . . .	...	...	2	1	3	...	0	33
16·0 Lf no phenol . . .	2	6	7	...	...	13	53	...
16·0 Lf with phenol . . .	...	4	2	...	...	0	67	...

2. *Distribution of toxoid between precipitate and filtrate.*

Wallace (1927) has shown that the addition of acid to toxoid, reducing the hydrogen ion concentration to pH 1·0, causes an insoluble precipitate to form. The rapid immunity index method was used to determine whether the precipitate was still antigenic. The results given in table XIII show that all the antigen is not removed from solution at pH 0·2 or pH 0·6 and that the suspensions of the insoluble precipitates in their original volume of buffer solution at pH 8·0 were as antigenic as the original toxoid.

TABLE XIII.

Showing the antigenic value of suspensions of insoluble acid precipitates of toxoid.

	Rapid index in days for 1·0 c.c.					
Original toxoid . . . . .	14	14	14	16	19	21
Suspension of hydrochloric acid precipitate formed at pH 0·2 . . . . .	14	14	19	...	...	...
Filtrate from precipitate at pH 0·2 readjusted to pH 8·0 . . . . .	16	16	19	...	...	...
Suspension of nitric acid precipitate formed at pH 0·6 . . . . .	12	14	16	...	...	...
Filtrate from precipitate at pH 0·6 readjusted to pH 8·0 . . . . .	16	16	19	...	...	...

3. Influence of various substances on toxoid.

(a) *Sodium ricinoleate*. Larson and Eder (1926) recommended for human immunisation diphtheria toxin detoxicated by the addition of sodium ricinoleate. Glenny and Pope (1927) have pointed out the advantage of commencing with diphtheria toxoid to avoid any possibility of recovery of toxicity that occurs if the soap is precipitated from a toxin soap mixture. Table XIV shows that both the immunity index and rapid index method have been used to confirm the antigenic value of diphtheria toxoid before and after the addition of sodium ricinoleate. The antigenic values of the two batches of toxoid used appear no worse and possibly slightly better after the addition of the soap.

TABLE XIV.

Showing the antigenic value of diphtheria toxoid before and after the addition of 2 per cent. sodium ricinoleate.

Dose.	Immunity index.										
	Toxoid.					Toxoid + soap.					
1·0 c.c. of batch A .	2	2	4	...	...	1	2	2	...	...	...
5·0 „ „ A .	1	2	2	...	...	1	1	...	...	...	...
	Rapid immunity index in days.										
1·0 c.c. of batch A .	22	24	30	...	...	22	22	22	...	...	...
5·0 „ „ A .	15	22	27	...	...	15	18	20	...	...	...
1·0 c.c. of batch B .	16	16	18	20	...	14	14	14	16	...	...
5·0 „ „ B .	14	14	14	16	19	14	14	14	14	14	16

(b) *Potassium alum*.—Glenny Pope Waddington and Wallace (1926, 1) showed that toxin or toxoid may be precipitated by the addition of varying quantities of potassium alum and that an emulsion of such a precipitate has high antigenic properties.

Table XV shows the relative antigenic values of a dilution of toxoid and a suspension of the precipitate produced by the addition of 2 per cent. potassium alum to the same toxoid and made up to the same volume. The results show that the presence of alum increases the antigenic value of toxoid; 1·0 c.c. of alum toxoid is as efficient as 5·0 c.c. of toxoid without alum.

TABLE XV.

*Showing the relative antigenic values of toxoid and a suspension of toxoid precipitated by alum.*

	Number of guinea-pigs showing an immunity index of—			Percentage number.	
	1.	2.	over 2.	1.	2 or better.
1·0 c.c. toxoid alone .	1	7	11	5	42
1·0 c.c. alum toxoid .	5	2	1	62	87
5·0 c.c. toxoid alone .	12	6	2	60	90
5·0 c.c. alum toxoid .	7	1	0	87	100

In a further experiment given in table XVI the immunity index was determined for 1·0 c.c. of toxoid diluted in saline and in 2 per cent. alum.

TABLE XVI.

*Comparing the antigenic value of 1·0 c.c. toxoid diluted with 4·0 c.c. saline and with 4·0 c.c. of 2 per cent. alum.*

Diluent.	Immunity index.
Saline . . .	2, 3, 3, 4, 4
2 per cent. alum .	1, 1, 2, 2

(c) *Turpentine and toluol.*—Glenny Waddington Wallace and Pope (1926, 2) have shown that the addition of turpentine or toluol increases the antigenic response of a guinea-pig to toxoid. Guinea-pigs were injected with varying doses of toxoid (8 Lf per c.c.) with the addition of 0·2 c.c. of turpentine or 0·5 c.c. toluol. Table XVII shows that 0·5 c.c. toxoid with the addition of turpentine or toluol was as effective as ten times that amount of toxoid alone.

4. *Toxoid - antitoxin floccules.*—Glenny and Pope (1927) have recommended the use of floccules prepared by the addition of antitoxin to toxoid for human immunisation. They also showed that the antigenic efficiency of the toxoid-antitoxin floccules was increased by

TABLE XVII.

*Showing the influence of addition of turpentine or toluol on the antigenic response of guinea-pigs to injection of toxoid.*

Dose of toxoid.	Addition.	Number of guinea-pigs showing an immunity index of—						Percentage number.			
		1.	2.	3.	4.	5.	Over 5.	1.	2.	3.	4 or better.
0·1 c.c.	None . . . . .	...	...	...	...	1	5	...	...	...	0
	0·2 c.c. turpentine	...	...	2	1	...	2	...	...	...	60
	0·5 c.c. toluol .	...	3	...	...	...	3	...	...	...	50
0·2 c.c.	None . . . . .	...	1	1	...	1	3	...	...	33	...
	0·2 c.c. turpentine	2	2	1	2	1	3	...	...	45	...
	0·5 c.c. toluol .	...	2	...	...	...	2	...	...	50	...
0·5 c.c.	None . . . . .	...	2	1	1	...	1	...	40	...	...
	0·2 c.c. turpentine	2	4	...	...	1	...	...	85	...	...
	0·5 c.c. toluol .	3	2	...	...	...	...	...	100	...	...
1·0 c.c.	None . . . . .	...	6	9	2	1	3	0	29	...	...
	0·2 c.c. turpentine	14	5	1	...	1	...	67	90	...	...
	0·5 c.c. toluol .	5	4	1	...	...	...	50	90	...	...
5·0 c.c.	None . . . . .	3	5	2	...	...	...	30	...	...	...
	0·2 c.c. turpentine	5	1	...	...	...	...	83	...	...	...
	0·5 c.c. toluol .	6	1	...	...	...	...	85	...	...	...

heat. Further experiments on the relative antigenic values of 15 batches of floccules prepared from both toxin and toxoid before and after heating to one hour at 70° C. are given in table XVIII. It will be seen that immunity is more rapidly produced by heated than by unheated floccules. There was no marked difference between the two types of floccules.

TABLE XVIII.

*Showing the relative antigenic value of toxin-antitoxin and toxoid-antitoxin floccules before and after heating.*

	Number of guinea-pigs showing an immunity index of—				Percentage number.	
	1.	2.	3.	over 3.	1.	2 or better.
Before heating . . . . .	7	2	4	14	29	33
After heating at 70° C. for 1 hour .	13	21	6	0	32	85

The same method has been used to demonstrate that some antigenic material is in a soluble form after a suspension of well washed floccules have been heated. Table XIX records a typical example of the results obtained.

TABLE XIX.

Showing the antigenic value of toxoid-antitoxin floccules and of the soluble and insoluble fractions after heating.

	Immunity index for dose of—					
	1·0 c.c.			5·0 c.c.		
Unheated suspension . . . . .	2	2	4	2	3	3
(a) Supernatant heated 1 hour at 80° C. . . . .	2	3	4	1	2	...
(b) Suspension . . . . .	1	2	2	1	3	3

5. Interference with immunity response.

(a) *With another antigen.* Glenny Hopkins and Waddington (1925) showed that rabbits made a lessened immunity response to the injection of diphtheria toxoid or toxin-antitoxin mixtures if at the time of injection the animal was already producing precipitin to horse serum. Glenny and Waddington (1926) showed that the addition of excess of scarlet fever toxin to diphtheria toxoid lessened the value of the latter as an antigen. The nonspecific interference between one antigen and another has also been demonstrated in guinea-pigs by means of the immunity index method. Guinea-pigs were injected with a toxin-antitoxin mixture given alone or at different intervals before or after the injection of from 0·5 to 5·0 c.c. of normal horse serum Table XX shows that a lessened immunity response occurred if serum was given one or two weeks before or at the same time as the toxin-antitoxin mixture but not if serum was given 7 days later or 4 weeks earlier.

TABLE XX.

Showing the antigenic response of guinea-pigs to the injection of 1·0 c.c. of a toxin-antitoxin mixture given alone or in conjunction with normal horse serum.

Normal horse serum.	Number of guinea-pigs showing an immunity index of—				Percentage number.	Conclusion.
	2.	3.	4.	Over 4.	4 or better.	
None given . . . . .	4	5	6	3	83	...
1·0 c.c. given 4 weeks before .	...	2	...	1	67	No interference.
“ “ 2 “ “ .	...	...	...	4	0	Interference.
“ “ 1 week “ .	2	3	...	13	27	“
“ “ at same time .	...	...	2	2	50	“
“ “ one week later .	1	3	...	...	100	No interference.

A similar experiment was made by injecting 5·0 c.c. of diphtheria toxoid alone or at the same time as, or 2, 4, and 6 days after the

injection of 0.5 c.c. normal horse serum. Table XXI again shows evidence of nonspecific interference.

TABLE XXI.

*Showing the antigenic response of guinea-pigs to diphtheria toxoid given alone or at the same time or after the injection of normal horse serum.*

	Number of guinea-pigs showing a rapid immunity index of—				Percentage number.
	12.	14.	16.	Over 16 days.	16 or better.
Toxoid alone . . . . .	1	2	3	3	67
Toxoid and normal horse serum .	...	1	2	8	25

(b) *By dye blockade.* Gay and Clark (1924) have shown that injection of trypan blue lessens or abolishes immunity response. Table XXII records the results of injecting 1.0 c.c. of a toxin-antitoxin mixture into 5 normal guinea-pigs and into 5 that had previously received six daily intraperitoneal injections of 1.0 c.c. of 1 per cent. trypan blue solution. The animals that had received the dye showed very little immunity response. It must be pointed out however that the lessened immunity response may have been due to the effect of the dye in lowering the condition of the guinea-pig and not to direct blockade of the reticulo-endothelial system.

TABLE XXII.

*Showing the antigenic response of normal and dye blockaded guinea-pigs to the injection of a toxin-antitoxin mixture.*

	Immunity index.				
Normal guinea-pigs . . . . .	2	3	3	4	7
Dye blockaded guinea-pigs	>5	7	12	13	20

6. *Evidence of dissociation by dilution of toxin-antitoxin mixture.*

Balanced mixtures of toxoid with three types of antitoxin were injected into guinea-pigs in doses of 0.001, 0.01, 0.1, and 1.0 c.c. diluted to 5 c.c. with saline, and in doses of 10 c.c. The animals were then Schick tested according to the immunity index method. These three types of antitoxin were "D" with an *in vivo* value three times that of the *in vitro* value, "E" with the two values equal, "F" modified antitoxin of Glenny (1913); such modified antitoxin always gives a lower *in vivo* than *in vitro* value when the two can be compared, although frequently no flocculation occurs even when the modified

antitoxin is blended with fresh antitoxin. Table XXIII shows that the same immunity response followed the injection of so wide a range of doses as 0·01 c.c. to 10 c.c. of mixtures containing antitoxin "D" and "E," while slightly better response followed the injection of 0·001 c.c. and 0·01 c.c. of mixtures of toxoid and modified antitoxin "F" than when larger doses were injected. Such results suggest that toxoid and antitoxin are dissociated when diluted.

TABLE XXIII.

*Showing the antigenic value of different dilutions of toxoid-antitoxin mixtures made with three types of antitoxin.*

Dose injected. Diluted to 5·0 c.c.	Immunity index for mixtures containing—											
	Antitoxin "D."				"E."				"F."			
0·001 c.c.	5	8	> 8	...	5	6	8	10	4	4	6	...
0·01 „	3	4	4	6	3	3	5	5	3	4	8	>11
0·1 „	3	4	5	9	5	5	...	...	6	9	...	...
1·0 „	3	5	>11	>11	3	...	...	...	7	...	...	...
Undiluted.												
10·0 c.c.	4	5	...	...	3	3	4	...	7	7	7	7

In the next experiment five mixtures were made with toxin or toxoid 30 fold over-neutralised with antitoxin. Of the five mixtures, two contained toxin, one with antitoxin "D" and the other with antitoxin "E"; the other 3 contained toxoid with all three types of antitoxin.

TABLE XXIV.

*Showing the antigenic value of different dilutions of 30 fold over-neutralised toxin or toxoid.*

Dose injected. Diluted to 5·0 c.c.	Number of guinea-pigs showing an immunity index of—						Percentage number immunised earlier than any controls (see table I).
	5.	6.	7.	8.	9 and 10.	Over 10.	
0·001 c.c.	1	2	3	3	3	5	35
0·01 „	0	2	4	1	2	4	46
0·1 „	3	1	0	5	1	6	25
1·0 „	0	0	0	2	2	12	0
Undiluted.							
10·0 „	0	0	0	1	1	9	0

Table XXIV gives a summary of results obtained by injecting dilutions of the various mixtures into guinea-pigs and testing their immunity by the routine method. At the time the tests were made a few of the immunity index control guinea-pigs were immune by the 8th week but none earlier. It will be seen from the table that none of the 11 guinea-pigs injected with 10 c.c. of over-neutralised mixtures was immune earlier than the controls nor were any of the

16 guinea-pigs injected with 1·0 c.c. mixtures diluted to 5·0 c.c. but that some immunity was produced by the injection of 0·001, 0·01 and 0·1 c.c. (diluted to 5·0 c.c. with saline) of mixtures of toxin or toxoid containing 30 times as much antitoxin as that required completely to neutralise all the toxin or toxoid present. The dilutions were made and the injection given within 2 hours of preparing the mixture.

These results show that greatly over-neutralised mixtures are not antigenic when given undiluted but suggest that high dilutions cause sufficient dissociation to enable some free toxin to act as a stimulus. No significant differences were seen between the five types of mixtures used.

These results were confirmed in a further experiment. Toxoid-antitoxin mixtures were prepared in which the antitoxin was 10 and 100 times the amount necessary completely to combine with the toxoid; control mixtures were made containing the same amount of normal horse serum containing no normal antitoxin. The proportion of toxoid to normal horse serum in these control mixtures was as 1 is to 0·3 and as 1 is to 3·0 in the 10 fold and 100 fold controls respectively. Tables XXV and XXVI record the immunity indices of guinea-pigs injected with different dilutions of the mixtures injected within 2 hours and also 7 days after the mixtures were made.

TABLE XXV.

*Showing the antigenic values of different dilutions of 10 and 100 fold over-neutralised toxoid in comparison with toxoid alone and toxoid to which normal horse serum (N.H.S.) has been added. Injections made with fresh mixtures.*

Injected. Diluted to c.c.	Immunity index.				
	10 fold over-neutralised.	100 fold over-neutralised.	Containing same volume of N.H.S. as 10 fold mixture.	Containing same volume of N.H.S. as 100 fold control.	Toxoid alone.
0·1 c.c.	9, >9, >12	>13, >33	11, 21, 27	19, >28	16, 16, 18
„	6, 7, 8	9, 17, 20	19	10, 12, 13, 18	15, 19, 33
„	6, 8, 9, 25	5, 17, 16	5, 7, 13	7, 20	2, 11, 22
„	6, 8, 19	12, 13, 21, 21	2, 2	7, 14, 17, 19	2, 2, 2, 2
„	>18, 19, 21	14, 22, >27	1, 1	1, 1	1, 1, 1, 2
1 c.c.	>16, >18, 21	12, >14, 26, >34	1, 1, 1	1, 1, 1	...

A number of conclusions can be drawn from the tables :

1. The degree of immunity induced by the injection of 1·0 c.c. of the toxoid is such that the interference caused by the injection of three times the volume of horse serum is not detected by the ordinary index method.
2. Excess of normal horse serum almost entirely suppresses the immunity response to 0·1 c.c. of toxoid.

3. 10 fold over-neutralised mixtures produced some immunity when injected in doses of 0·001, 0·01 and 0·1 c.c. diluted to 5·0 c.c. shortly after preparation.
4. The presence of excess antitoxin increases the antigenic value of small doses of toxoid, probably due to the effect of slow dissociation spacing out the stimulus.
5. 100 fold over-neutralised mixtures produce some immunity in doses of 0·01 c.c. diluted to 5·0 c.c. shortly after the preparation.
6. Scarcely any dissociation occurs when over-neutralised mixtures are diluted 7 days after mixing.

TABLE XXVI.

*Showing the antigenic values of different dilutions of 10 fold over-neutralised toxoid in comparison with toxoid alone and toxoid to which normal horse serum has been added. Injections made 7 days later.*

Dose injected. Diluted to 5·0 c.c.	Immunity index.		
	10 fold over-neutralised.	Containing same volume of N.H.S. as 10 fold mixtures.	Toxoid alone.
0·0001 c.c. .	16, 18, 20, 22	16, 17	11, >15, 24
0·001 „ .	7, 12, 17	7, 11, 13	13, 13, 16
0·01 „ .	17, >17, 25	6	4, 5, 9
0·1 „ .	10, 12, 15, 21	2, 2, 3, 4	1, 2, 2, 2
1·0 „ .	7, >16, 18	1, 3	1, 1, 1, 1
Undiluted. 10·0 c.c. .	18	1	...

## SUMMARY.

1. Guinea-pigs receiving weekly injections of Schick toxin may become Schick negative at the eighth injection.

2. Guinea-pigs receiving weekly injections of Schick toxin are more readily immunised when the series of injections is commenced in summer or early autumn.

3. The general condition of animals affects their rate of immunisation

4. The immunity index method has been used to demonstrate—

(a) that qualitative differences exist between different batches of toxoid depending upon (1) amount of precipitable material (2) types of broth use in preparation (3) amount of formaldehyde used (4) presence or absence of phenol:

(b) that toxoid precipitated at pH 0·2 is still antigenic:

(c) that ricinoleate toxoid is antigenic:

(d) that the addition of potassium alum or of turpentine or toluol increases the antigenic efficiency of toxoid:

- (e) that the antigenic efficiency of toxin-antitoxin and of toxoid-antitoxin floccules is increased by heating the floccules to 70° C. for one hour:
- (f) that immunity response to one antigen may be lowered by simultaneous response to other antigens:
- (g) that dye-blockaded guinea-pigs show little response:
- (h) that dilution of toxin-antitoxin mixtures causes dissociation.

## REFERENCES.

- GAY, F. P., AND CLARK, A. R. . . . *Journ. Amer. Med. Assoc.*, 1924, lxxxiii. 1296-7.
- GLENNY, A. T. . . . . *J. Hyg.*, 1913, xiii. 63.
- GLENNY, A. T., AND ALLEN, K. . . . *J. Hyg.*, 1922, xxi. 104.
- GLENNY, A. T., ALLEN, K., AND HOPKINS, B. E. . . . *Brit. J. Exper. Path.*, 1923, iv. 19.
- GLENNY, A. T., HOPKINS, B. E., AND WADDINGTON, H. . . . this *Journal*, 1925, xxviii. 305.
- GLENNY, A. T., AND POPE, C. G. . . . this *Journal*, 1927, xxx. 587.
- GLENNY, A. T., POPE, C. G., WADDINGTON, H., AND WALLACE, U. (1) . . . this *Journal*, 1926, xxix. 31.
- GLENNY, A. T., POPE, C. G., WADDINGTON, H., AND WALLACE, U. (2) . . . *Chemistry and Industry*, 1926, xlv. 415.
- GLENNY, A. T., AND WADDINGTON, H. . . . this *Journal*, 1926, xxix. 118-122.
- HARRIES, E. H. R. . . . . *Proc. Roy. Soc. Med.*, 1927, xxi. 197.
- KELLOGG, W. H., AND STEVENS, I. M. . . . *Journ. Amer. Med. Assoc.*, 1927, lxxxix. 273.
- LARSON, W. P., AND EDER, H. . . . *Journ. Amer. Med. Assoc.*, 1926, lxxxvi. 998.
- MOLONEY, P. J., AND WELD, C. B. . . . this *Journal*, 1925, xxviii. 655.
- D'BRIEN, R. A. . . . . this *Journal*, 1926, xxix. 320.
- OPITZ, H. . . . . *Klin. Woch.*, 1927, vi. 1701.
- WALLACE, U. . . . . this *Journal*, 1927, xxx. 667.
- WATSON, A. F., AND WALLACE, U. . . . this *Journal*, 1924, xxvii. 289.

